

## Metabolism of Toxic Pyrrolizidine Alkaloids from Tansy Ragwort (*Senecio jacobaea*) in Ovine Ruminal Fluid under Anaerobic Conditions†

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The ability of ovine ruminal fluid to metabolize pyrrolizidine alkaloid (PA) from *Senecio jacobaea* under anaerobic conditions was evaluated. Four fistulated sheep fed PA served as individual sources of ruminal fluid, which was incubated in a defined minimal salts medium under two different anaerobic conditions, denitrifying and methanogenic. Anaerobic cultures amended with ovine ruminal fluids (20%), PA (100 µg/ml), and a defined minimal salts medium were monitored for a period of several days. These cultures revealed that while PA was not depleted in sterile, autoclaved controls or under denitrifying conditions, it was metabolized during periods of active methanogenesis under methanogenic conditions. In addition, samples of ruminal fluid were separated by differential centrifugation under anaerobic conditions, and the resultant supernatants were tested for their ability to metabolize PA as compared with those of the respective uncentrifuged control fluids. Uncentrifuged controls exhibited a PA depletion rate of  $-4.04 \pm 0.17$  µg of PA per ml per h. Supernatants 1 (centrifuged at  $41 \times g$  for 2 min), 2 (centrifuged at  $166 \times g$  for 5 min), and 3 (centrifuged at  $1,500 \times g$  for 10 min) exhibited significantly slower depletion rates, with slopes of data representing  $-1.64 \pm 0.16$ ,  $-1.44 \pm 0.16$ , and  $-1.48 \pm 0.16$  µg of PA metabolized per ml per h, respectively, demonstrating no statistically significant difference among the supernatant cultures. Microscopic evaluations revealed that protozoa were present in the control whole ruminal fluid and to a lesser extent in supernatant 1, while supernatants 2 and 3 contained only bacteria. Supernatant 4 (centrifuged at  $1,900 \times g$  for 20 min), from which all visible microflora were removed, exhibited no PA depletion during the course of incubation. The microbial population common to all supernatants and the control ruminal fluid were the small bacteria. These data suggest that the metabolism of PA can be mediated by a consortium (or single isolate) of small bacteria in the ovine rumen.

Pyrrolizidine alkaloids (PA) are the principle toxic components in tansy ragwort (*Senecio jacobaea*) and a number of other toxic plants throughout the world (3, 4), and consumption of PA from these plants constitutes the major economic loss of livestock in the western United States (11). In general, sheep are resistant to this toxin, while cattle and horses are not (12, 17). Although it is not entirely clear what specific host factors confer resistance and/or susceptibility, recent evidence suggests that the primary protective mechanism in vivo is localized in the anaerobic ruminal microflora (10, 23). The role bacteria play in species susceptibility to PA toxicosis was addressed by Lanigan, who isolated an obligate anaerobic *Peptococcus* sp. which grew on heliotrine (25). While these results suggested that a PA could be dissimilated in the anaerobic rumen, it raised additional questions regarding the following: (i) the susceptibility of other PAs, like seneciocine and jacobine (from *S. jacobaea*), to dissimilation; (ii) the potential for PA metabolism under various anaerobic conditions (methanogenic and denitrifying, for example); and (iii) the diversity of bacteria which possess the capability to detoxify PAs. Since PAs are not intrinsically toxic until converted to activated pyrroles (3) or trans-4-hydroxy-2-hexenal by the liver (32), rapid metabolism by ruminal bacteria would disallow higher liver burdens and thus serve as a mechanism for detoxification in sheep (10).

The purpose of this study was (i) to evaluate if PA from *S. jacobaea* could be anaerobically metabolized in the ruminal contents of sheep, (ii) to evaluate what anaerobic electron-accepting conditions facilitate PA metabolism, and (iii) to examine which microbial fraction of the ruminal contents is responsible for the activity. On the basis of an approach employed by Blackburn and Hobson (2), a series of differential centrifugation experiments was designed to anaerobically fractionate ruminal contents, separating out bacteria from various protozoa, and then to examine the resultant supernatants to quantitate their capabilities to metabolize PA in vitro.

### MATERIALS AND METHODS

**Animals and rumen collection techniques.** Four adult sheep with permanent ruminal fistulas were the sources of ruminal fluid. Three of the animals were Rambouillet wethers, and one was a Suffolk ewe. The animals were kept in confined indoor pens and fed alfalfa pellets containing 10% tansy ragwort (*S. jacobaea*) ad lib. Ruminal contents were obtained from the dorsal ruminal sac immediately after removing the fistula plug. These contents were then strained through nylon netting into an insulated thermos, completely filling the container to eliminate air pockets. The samples were transported to the laboratory within 30 min, where they were restrained through nylon netting into either a pre-warmed Erlenmeyer flask purged with N<sub>2</sub>-CO<sub>2</sub> (70%:30%) capped with an airtight stopper or dispensed directly into

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serum bottles amended with one of three anaerobic media. A stock container of ruminal fluid was kept in a 37°C incubator under a continuous gas purge, with additional samples being removed as necessary. After each sample was removed, the flask was flushed with N<sub>2</sub>-CO<sub>2</sub>.

**Defined anaerobic medium preparations.** One of three different medium preparations was employed to study the biodegradability of the PA. In the centrifugation experiments, only McDougalls buffer was used, whereas for the experiments on the effects of anaerobic conditions on PA metabolism, defined denitrifying and methanogenic media, as well as McDougalls buffer, were used on the basis of the protocols described elsewhere (27, 28). McDougalls buffer was used because it roughly simulates the composition and buffering capacity of sheep saliva (26). Preliminary experiments showed that after a couple of days of incubation, ruminal fluid alone would demonstrate a drop in pH from approximately 6.5 to 5.7 in the absence of buffer. A 50% dilution of ruminal fluid in McDougalls buffer in six pilot studies revealed a pH change of only 0.2 pH unit over 48 h of incubation. Mean pH values in McDougalls buffer were approximately 6.7 to 6.8.

All medium preparations are described in detail elsewhere (27–29) and only briefly discussed here. McDougalls buffer was prepared by boiling approximately 1.3 liters of distilled water for approximately 20 min and then cooling it to room temperature while simultaneously purging it with an N<sub>2</sub>-CO<sub>2</sub> (70%:30%) gas stream for approximately 30 min. The following salts were then added (in grams per liter) while the flask was purged with gas: NaHCO<sub>3</sub>, 9.8; KCl, 0.57; Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O, 9.3; NaCl, 0.47; MgCl<sub>2</sub>, 0.06; CaCl<sub>2</sub>, 0.04; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.64 (26). After the medium was prepared, 160-ml serum bottles purged with N<sub>2</sub>-CO<sub>2</sub> were amended with 50 ml of anaerobic medium, 50 ml of fresh ovine ruminal fluid, and a 100-μg/ml concentration of crystalline PA extracted from the dried tansy ragwort plant *S. jacobaea* by the method of Craig et al. (12). This concentration (i.e., 100 μg/ml) was calculated to represent the approximate quantity of PA that would be consumed by an animal in the field. After all inoculations and additions were made, all bottles were sealed with butyl rubber stoppers and aluminum crimps and depressurized to 1 atm (1 atm = 101.29 kPa).

Methanogenic and denitrifying media were prepared in a similar manner, except that the compositions of the headspace and salts were different. For the denitrifying medium, after the distilled water was boiled, the flask was cooled under a flow of argon gas and the following components were added: 29 mM Na<sub>2</sub>HPO<sub>4</sub>, 19 mM KH<sub>2</sub>PO<sub>4</sub>, 5.6 mM NH<sub>4</sub>Cl, 0.4 mM MgSO<sub>4</sub> · H<sub>2</sub>O, 30 mM KNO<sub>3</sub>, 9 mM FeSO<sub>4</sub> · H<sub>2</sub>O, 1.2 mM CuSO<sub>4</sub> · H<sub>2</sub>O, and finally 15- and 10-ml aliquots from concentrated stocks of trace elements and vitamins, respectively (27–29). After the water was boiled for the methanogenic medium, the flask was cooled to room temperature and purged with a 70%:30% N<sub>2</sub>-CO<sub>2</sub> gas mixture passed over a reduced R3-11 catalyst to remove traces of oxygen. To 1 liter of distilled water, the following additions were made from prepared stock solutions: 1 ml of the redox indicator resazurin (3.9 mM stock), 1.57 ml of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (20 mM stock), 1 ml of FeCl<sub>2</sub> · 4H<sub>2</sub>O (1.86 mM stock), 1 ml of the reductant Na<sub>2</sub>S · 9H<sub>2</sub>O (2.3 mM stock), 2.6 g of NaHCO<sub>3</sub>, and finally 10- and 15-ml aliquots of trace elements and vitamins, respectively (27–29). After the media were prepared, 80 ml was dispensed into 160-ml serum bottles purged with either argon (denitrifying cultures) or N<sub>2</sub>-CO<sub>2</sub> (methanogenic cultures), and the bottles were each inoculated with 20 ml of the ruminal fluids, capped with a butyl rubber

stopper and an aluminum crimp, and depressurized to 1 atm. PA additions were made from a concentrated anaerobic stock solution. Background control cultures were established in a similar manner, except that no PA was added to the culture. All cultures were established in triplicate and incubated in the dark at 37°C. Sterile controls were prepared by autoclaving the cultures two to three times per day for 3 days and then amending them with a sterile aliquot of PA and vitamins (Millipore; 0.22-μm-pore-size sterile filters). All sampling and additions of PA were made by using standard anaerobic technique.

**Analytical methods for quantification of PA.** For the centrifugation experiments, 5-ml aliquots of sample were routinely obtained during the course of incubation by using anaerobic technique. Approximately 200 μl of 50% phosphoric acid was added to each sample to stop microbial activity and frozen at –10°C if necessary. A modified high-performance liquid chromatography (HPLC) analysis was used to quantify the PA concentration (30). HPLC equipment consisted of an SP8775 autosampler, a column (15 cm by 4.1 mm) with 5-μm packing, a Beckman binary gradient pump system with two model 110A pumps and a model 421 pump controller, and a Beckman model 165 UV detector set at 214 nm and 0.1 absorbance units, full scale. Data were collected with a Perkin-Elmer LCI-100 integrator connected to a model 7500 data station. An acid-base extraction procedure was used to extract the PA. One milliliter of an internal standard, monocrotaline (0.2 μg/ml; 1% phosphoric acid), and 4 ml of methylene chloride were added, and the samples were vortexed for 10 s. The samples were then centrifuged at 1,000 × g for 5 min, and the methylene chloride layer was removed and discarded. The pH of this solution was adjusted to 10.5 to 12.00 by using 10 M NaOH, and an additional 4 ml of methylene chloride was added. Samples were vortexed for 10 s and centrifuged, and the methylene chloride layer was removed and evaporated to dryness. The residue was resolubilized in 2 ml of HPLC-grade methanol, and an volume of 50 μl was injected onto the HPLC column for PA analysis.

In the biodegradation experiments, a rapid thin-layer chromatographic method was employed for PA analysis. Aliquots (1 to 5 ml) of sample were alkalized to pH 11 by using 10 M NaOH and mixed on a vortex mixer. Approximately 3.5 ml of dichloromethane was added to each sample, and the samples were again mixed on a vortex mixer for 30 to 60 s. The dichloromethane layer was removed from the sample vial and evaporated under a flow of argon to dryness. After drying, approximately 100 μl of dichloromethane was added, mixed, and spotted (2 μl) onto high-performance thin-layer chromatography plates (Whatman, Clifton, N.J.). High-performance thin-layer chromatography plates were placed in a developing chamber and chromatographed with a solvent composed of 72% chloroform, 18% methanol, and 10% propionic acid (by volume) for approximately 30 min. After the solvent line reached the top, the plates were removed from the chamber and developed with Dragendorff's reagent, after which the PA spots appeared light pink. This treatment was followed by several light coats of 5% sodium nitrite and cupric chloride, after which the PA spots appeared dark red to brown. Photographs were made of each plate after development.

**Centrifugation fractionation experiments.** A differential centrifugation scheme adapted from Blackburn and Hobson (2) was used to separate whole ruminal fluid into four fractions of different sizes containing different combinations of microorganisms. A total of 15 individual trials were

successfully completed over 3 months, utilizing ruminal fluid from one of four animals: sheep no. 373 ( $n = 7$ ), no. 374 ( $n = 2$ ), no. 376 ( $n = 2$ ), and no. 378 ( $n = 4$ ). Comparisons among animals and among supernatant fractions were made. Each trial was accomplished as follows: four 50-ml aliquots of fresh ruminal fluid from a single sheep were transferred into prewarmed  $\text{CO}_2$ -flushed polypropylene centrifuge tubes, and the tubes were anaerobically sealed. The centrifuge speeds for all samples were  $41 \times g$  for 2 min (supernatant 1),  $166 \times g$  for 5 min (supernatant 2),  $1,500 \times g$  for 10 min (supernatant 3), and  $19,000 \times g$  for 20 min (supernatant 4) in an ultracentrifuge (Beckman J2-21 with a JA-17 rotor). After centrifugation, the supernatant volume was measured and 50 ml was placed into prewarmed ( $37^\circ\text{C}$ )  $\text{CO}_2$ -flushed 160-ml serum bottles. A volume of 50 ml of anaerobically prepared McDougalls media was then transferred to each degassed serum bottle, and the bottles were purged for an additional 15 to 20 min before being sealed with a butyl rubber stopper and an aluminum crimp. With a 50% (vol/vol) McDougalls buffer-ruminal fluid mixture, the pH in all trials changed by an increase of only 0.1 over the entire 48-h incubation period. Mean pH values at time zero were 6.7 to 6.8 after 48 h. A 50-ml aliquot of whole ruminal fluid (uncentrifuged), plus 50 ml McDougalls buffer, served as a positive control for each trial. Crystalline PA was added to each serum bottle at a concentration of  $100 \mu\text{g/ml}$  prior to sealing the bottle with a rubber septum. All cultures were then placed in an environmental shaker at  $37$  to  $39^\circ\text{C}$ , and the shaker was flushed for an additional 15 to 20 min with 100%  $\text{CO}_2$  via a temporary 21-gauge needle in the septum. Aliquots (5 ml) of the media were removed for HPLC analysis after  $\text{CO}_2$  flushing (time, 0 h) and at 12, 24, and 48 h by using the anaerobic Hungate technique.

**Microscopic evaluation of control ruminal fluid and supernatants.** Evaluation of the whole ruminal fluid and prepared supernatants for size and type of microorganisms present was accomplished by using a Zeiss light microscope equipped with a phase contrast. Hucker's modification of the Gram stain for anaerobes was done, and wet mounts were used for phase microscopy. Approximate numbers and size of protozoa and of large and small bacteria were estimated in each sample.

**Analysis of experimental error.** The potentially deleterious effects of ultracentrifugation on the PA-metabolizing consortium and the rate of depletion were assessed. The depletion rate of whole, uncentrifuged ruminal fluid in each trial served as the positive control. One sheep (no. 373) was utilized on three occasions and another sheep (no. 378) was used on one occasion as the source of ruminal ingesta. Whole ruminal fluid was collected, processed, and centrifuged as described above. Two aliquots were prepared in parallel for each centrifugation speed. After centrifugation, one set of supernatants was poured off and immediately remixed with its respective pelleted material by using a vortex mixer. The other supernatant was amended into the anaerobic serum bottles with McDougalls buffer as described above. Sampling, extraction, and HPLC analysis were done for each culture as discussed above.

The effects of anatomical sampling sites on PA disappearance were also assessed in parallel with the centrifugation experiments. Nonhomogeneity of ruminal ingesta within various compartments of the rumen was considered a possible source of error. This trial compared the depletion rates of ruminal fluid from the following two sites: (i) a superficial site located just below the level of the ruminal fistula in the dorsal sac of the rumen, and (ii) a site deep in the ventral

cranial sac of the rumen. Trials were run utilizing two sheep (no. 376 and 378). Sample handling, incubation, and analyses were identical to those described above. Additionally, the effects of sampling time from the sheep were also assessed. The effects of feeding and fasting as they relate to population dynamics of the ruminal consortium were considered to be another parameter which may have affected culture variability. Two sheep (no. 373 and 378) were used for these trials. The sheep were fed their usual daily ration of tansy-containing feed at 8:00 a.m. after an overnight fast. Any remaining feed was taken away after 1 h. Samples of ruminal fluid were taken prior to feeding and at 1, 3, and 5 h postfeeding. Samples were processed as described above, except that only PA depletion rates of whole ruminal fluid were determined and compared.

## RESULTS

**Effects of centrifugation, rumen site, and sampling time on PA metabolism.** No statistical differences were found between the PA depletion rates of the uncentrifuged whole ruminal fluid control and the resuspended pellets. With immediate resuspension of each of the ruminal pellets (supernatants 1 through 4), the PA depletion rates of the resuspended cultures had a mean PA depletion rate equivalent to that observed in the uncentrifuged control fluid. Those aliquots of supernatants that were not resuspended produced rates similar to that observed in the 15 trials (Fig. 1 and Table 1, supernatants 1 through 4).

Additionally, the timing of the sampling relative to feed intake did not alter the rate of PA metabolism in the ruminal fluid. The selected times of sampling bracketed the usual morning sampling times used for the ultracentrifugation experiments. Similarly, there was no evidence that sampling from different anatomical sites within the ruminal sac of the animal produced any significant difference in the net rate of PA depletion in the two animals studied. The coefficients of variation for the extraction and HPLC analysis of PA in ruminal fluid were found to be 4.6% for the  $100\text{-}\mu\text{g/ml}$  concentration of PA and 5.3% for the  $10\text{-}\mu\text{g/ml}$  concentration of PA.

The results of the microscopic examination (phase-contrast and light microscopies) and semiquantification of microorganisms present in the supernatants are shown in the last column of Table 1. Large numbers of protozoa were present in the uncentrifuged whole ruminal fluid control, while approximately 75% were pelleted out in supernatant 1 ( $41 \times g$  for 2 min). Protozoa were totally absent in supernatants 2 through 4. The predominant microorganisms in supernatants 1 through 3 were bacteria, but in supernatants 2 and 3 decreased densities of bacteria were evident compared with those of the control and supernatant 1. Supernatant 4 had few to no microorganisms evident under light or phase-contrast microscopy at time zero. However, subsequent culture of supernatant 4 produced turbidity in McDougalls buffer after 48 h, suggesting that although the medium demonstrated no significant rate of PA depletion during the first 48 h, the medium was not sterile. After 48 h of incubation, the microscopic observations in supernatant cultures 1 through 3 were found to be similar to that recorded at the initiation of the experiment.

**Centrifugation fractionation experiments.** In the 15 trials, the majority (13 of 15) of the control samples (uncentrifuged whole ruminal fluid) had metabolized the PA in 24 h when amended with  $100 \mu\text{g}$  of PA per ml (Fig. 1). In one trial, ruminal metabolism of PA took 48 h (sheep no. 373), and in

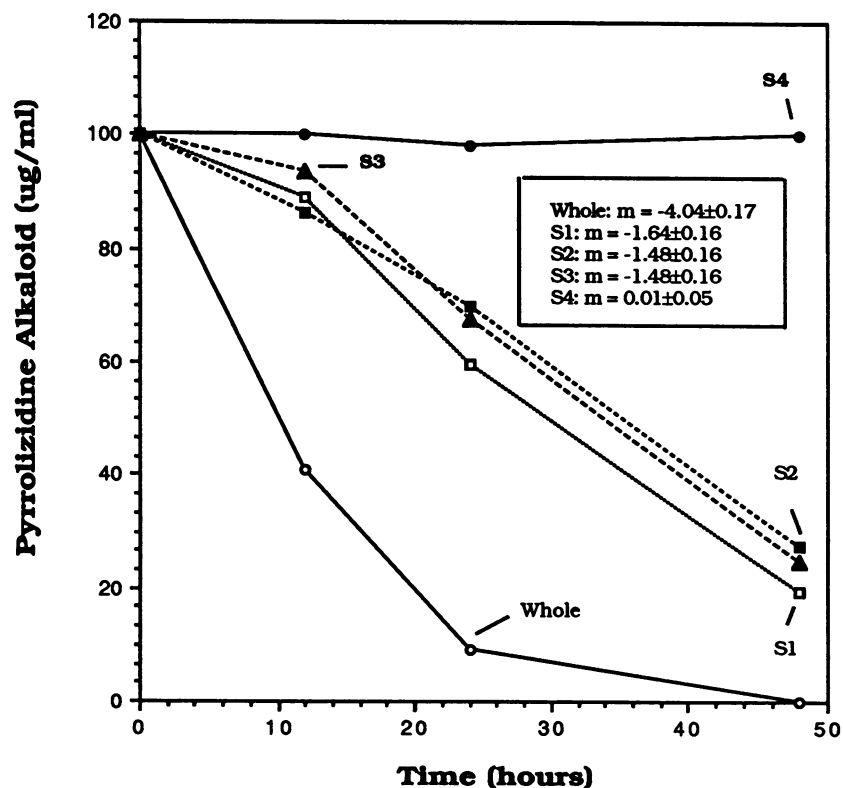


FIG. 1. Concentrations of PA remaining after in vivo incubation with ovine ruminal fluid. Slopes of data represent rates of metabolism equivalent to micrograms of PA metabolized per milliliter per hour. S, supernatant. For details, see the text.  $m$  is the slope (metabolism equivalent).

the other trial, no substrate depletion was observed. In the control samples (whole ruminal fluid), the mean PA concentration remaining after 12 h of incubation was approximately 41% of the original amendment (Table 1). In all cases, pasteurization, autoclaving, and high-speed ultracentrifugation at  $19,000 \times g$  for 20 min ablated biological activity within this time frame.

Cultures established with supernatants 1 through 3 consistently took longer to detoxify the PA relative to the uncentrifuged whole ruminal fluid, while those cultures initiated with supernatant 4 demonstrated no PA metabolism within the 48-h incubation period (Fig. 1 and Table 1). The rates of PA depletion were analyzed by linear regression. The whole ruminal fluid samples metabolized PA at a very rapid rate,

TABLE 1. PA concentrations (means  $\pm$  standard deviations) in control (whole) and fractionated ovine ruminal fluid over 48 h of incubation and microbiological characterization of whole ruminal fluid and supernatants as determined by light and phase-contrast microscopy at time zero

Ruminal fluid (no. of replicates)	PA concn ( $\mu\text{g/ml}$ )				Microbiological characterization
	0 h	12 h	24 h	48 h	
Whole (14)	100.7 $\pm$ 1.9	41.0 $\pm$ 18.4	9.2 $\pm$ 12.3	0.1 $\pm$ 0.3	All shapes and sizes of bacteria are present, including gram-negative rods and cocci; large and protozoa are abundant.
Supernatant 1 (13)	101.5 $\pm$ 1.5	89.1 $\pm$ 6.1	59.7 $\pm$ 16.4	19.3 $\pm$ 28.5	All shapes and sizes of bacteria are present; large fragments of debris and large protozoa are absent.
Supernatant 2 (15)	102.8 $\pm$ 1.3	86.3 $\pm$ 18.2	69.9 $\pm$ 18.2	27.5 $\pm$ 29.8	All shapes and sizes of bacteria are present; there is a subjective decrease in the overall concentration of microorganisms; protozoa are absent.
Supernatant 3 (15)	101.8 $\pm$ 2.1	93.8 $\pm$ 8.2	67.5 $\pm$ 26.5	24.7 $\pm$ 28.7	Protozoa and large bacteria are absent; small (–) rods and undifferentiated debris predominate; some gram-negative bacteria are present (<1% overall total).
Supernatant 4 (14)	100.3 $\pm$ 7.8	100.3 $\pm$ 7.8	98.3 $\pm$ 9.3	100.2 $\pm$ 7.4	Protozoa, large bacteria, and small bacteria are absent; undifferentiated debris stained with safarnin is abundant

TABLE 2. Anaerobic metabolism or biotransformation of different concentrations of PA under denitrifying and methanogenic conditions

Medium	Time required for complete PA disappearance at PA concn of <sup>a</sup> :				
	50 μg/ml	100 μg/ml	500 μg/ml	1,000 μg/ml	2,000 μg/ml
Denitrifying	NBD <sup>b</sup>	NBD	NBD	NBD	NBD
Methanogenic	24 h	24 h	48 h	7 days	NBD
McDougalls	24 h	24 h	48 h	7 days	NBD

<sup>a</sup> In all cases, autoclaved sterile controls failed to metabolize the amended PA.

<sup>b</sup> NBD, not biodegraded.

$-4.04 \pm 0.17$  μg of PA per ml per h, when compared with the rates for supernatants 1 through 3, which were  $-1.64 \pm 0.16$ ,  $-1.44 \pm 0.16$ , and  $-1.48 \pm 0.16$  μg of PA per ml per h, respectively. The rates of PA depletion in the supernatants were not statistically different from each other, whereas the rate observed in the whole ruminal fluid control sample was statistically different from those of supernatants 1 through 3 ( $P < 0.05$ ). Supernatant 4 produced a depletion rate of  $0.01 \pm 0.05$  μg of PA per ml per h, which was significantly different from those of both the control samples and supernatants 1 through 3 ( $P < 0.001$ ).

**Effects of different bacterial media and electron acceptors on PA depletion.** Table 2 qualitatively summarizes the observations made on the depletion of PA in various anaerobic media. These results demonstrate that no PA depletion was observed in sterile control cultures or in denitrifying cultures. Conversely, PA depletion was noted in less than 24 h for all cultures amended with 100 μg or less of PA per liter in both McDougalls buffer and methanogenic medium. In each of the latter two culture conditions, active methanogenesis was observed during PA depletion, and successive refeeding of these cultures with PA revealed a disappearance of the amended PA in less than 48 h.

## DISCUSSION

In all in vitro experiments conducted, incubation of whole ovine ruminal fluid with PA resulted in the rapid metabolism, i.e., in 12 to 24 h, of the toxin as compared with only minimal metabolism by ruminal fluid of the susceptible bovine species (10, 11).

Rapid metabolism of PA was observed under anaerobic conditions in both whole and fractionated ovine ruminal fluids. These data demonstrate that whole ruminal fluid produced the fastest rate of PA metabolism, while the fractionated supernatants obtained through ultracentrifugation produced significantly slower rates, which is most likely attributed to the adsorption of organisms onto plant debris. Additionally, there was no statistical difference among the rates observed in supernatants 1 through 3 nor among the individual sheep ( $P > 0.05$ ). This may suggest that the active biomass responsible for the observed activity is relatively constant throughout all supernatants studied. Since the rate of PA depletion did not significantly change with the removal of the smaller protozoa (supernatant 2) or large bacteria (supernatant 3), it is suspected that the group or consortium of bacteria responsible for PA metabolism is the group of smaller ruminal bacteria. This particular centrifuge fractionation scheme has been used previously for separating bacteria by size and to quantitate the physiological properties of

various fractions of ruminal fluid (2). The microscopic observations made in this study (Table 1) agree with those of previous studies.

Since the centrifugation process did not alter the ability of ruminal fluid to metabolize the compound, the faster rate of metabolism seen in whole ruminal fluid controls must be attributed to factors removed or reduced in the centrifugation process. Large protozoa may represent one factor responsible for the observed differences, since they are readily removed by the centrifugation process. Since the rumen is a unique ecosystem in which the microbial mass responsible for ruminal fermentation consists of both bacteria and protozoa, a synergism between protozoa and bacteria may exist that enhances PA metabolism. While the presence of protozoa is not essential for the growth of sheep (1, 35), the ability of protozoa to attack major components of feedstocks suggests that they may serve an important role in the fermentation and stabilization of the ruminal ecosystem (6, 7). In addition, it has been reported that the absence of protozoa can change the dominant types of bacteria (8, 14, 22), although longer periods of incubation and adaptation are likely to be required. Protozoa are also important predators of certain bacteria in the rumen and can play an important role in the microbial ecology of the rumen (7, 8). Another factor that could account for a decrease in the rate of disappearance includes a loss in the numbers of bacteria due to their adherence to protozoa and pelleted plant particles. Since it has been shown that many bacteria adsorb onto plant debris in the rumen, it is likely that the reduced rates of PA depletion in supernatants 1 through 3 can be attributed in part to reduced bacterial biomass. Additionally, the loss of particular energy-yielding carbon sources, nutrients, and/or cofactors with the plant debris may account for some degree of diminished bacterial activity.

Table 2 reveals that PA was metabolized only in either the McDougalls buffer or the methanogenic medium and not in the denitrifying medium. Additionally, it appears that there may be a toxic threshold above which metabolism of PA cannot be demonstrated. It can be seen in Table 2 that PA at concentrations greater than 500 μg/ml requires increasing periods of incubation before its disappearance and that PA concentrations in excess of 1,000 μg/ml may be too toxic for metabolism to occur. In both McDougalls buffer and methanogenic medium, there was an abundance of bicarbonate-CO<sub>2</sub> and active methanogenesis was noted, while in the denitrifying medium, there was no methanogenesis. These data suggest that the degradation of PA may be dependent, at least to some extent, upon the fermentative organisms in the methanogenic consortium or the methanogens. Some studies have shown that the inhibition of methanogenesis with chloral hydrate or halogenated methanes actually enhanced heliotropine metabolism (24). These results suggest that PA-metabolizing organisms may compete with methanogens for reducing equivalents and that excess electron donors may augment the reductive fractions involved in PA dissimilation (23, 24, 31). The lack of PA activity in the denitrifying consortium may be due to the fact that either (i) denitrifying bacteria do not metabolize PA or (ii) the presence of nitrate inhibited the methanogenic consortium such that it could not metabolize the amended PA.

In an attempt to assess the stoichiometry of metabolism and the extent of mineralization under these biodegradative conditions, an average molecular formula and molecular weight were calculated for the mixture of alkaloids known to be present in tansy ragwort. PA are actually a composite of many different alkaloid structures, which in the case of *S.*

*jacobaea* consists of alkaloids such as jacobine, jacozone, jacoline, senecionine, and seneciophylline. The following equation presents the mass balance for an average molecular formula (average molecular weight =  $349.62 \pm 15.5$ ) of PA under methanogenic conditions:  $C_{18} \pm 0 H_{25.2} \pm 1.9 N_1 \pm 0 O_{5.8} \pm 0.83 \cdot 9.5 H_2O \rightarrow 7.74 CO_2 + 10.25 CH_4 + NH_3$ . This reaction shows that for every millimole of PA, approximately 10.25 mmol of methane will be generated, assuming complete mineralization. At a concentration of approximately 100  $\mu$ g/ml, and by using an average molecular weight of 349.62 g/mol, there is approximately 2.86 mmol of PA present in the system. This quantity of PA could produce approximately 30 mmol of  $CH_4$  per 100 ml of culture. Comparison of cultures examined under methanogenic conditions in the presence of PA revealed no significant enhancement or inhibition of methane production relative to the background control (i.e., with no PA amendment) cultures. These data suggest that while the PA are being metabolized or biotransformed under methanogenic conditions, there is no mineralization of the PA and that the majority of the parent carbon compound is likely to exist in the form of some as-yet-unidentified intermediate(s).

The ability of anaerobic bacterial consortia to metabolize toxic and/or xenobiotic molecules has been well documented and has important implications in the fate of these contaminants in the environment. Many toxic and environmentally important pesticides and plant derivatives (lignin monomers), which were previously thought to be recalcitrant, are now known to be metabolized by many diverse groups of anaerobic organisms (5, 15, 16, 18, 28, 31, 33, 34, 36). As early as 1957, it was observed that bovine ruminal organisms were capable of degrading various organophosphate pesticides in vitro (9). In yet another example of the ability of the rumen to detoxify organic substrates, it was found that mimosine, a toxin found in the leguminous shrub *Leucaena leucocephala*, undergoes ruminal dissimilation to a nontoxic metabolite in toxin-resistant goats which inhabit the Hawaiian islands (19, 20). Cross-inoculation of susceptible Australian goats with Hawaiian goat ruminal bacteria confers toxin resistance to the former goat populations (21).

A structurally analogous molecule to the PA found in tansy ragwort is heliotrine, produced from the plant *Heliotropium europaeum*. Heliotrine is a PA that has been found to be reduced to a nonhepatotoxic form in sheep rumen (13). In vitro studies have documented that residual bacteria in strained ruminal fluid from resistant sheep are capable of degrading the added heliotrine when incubated anaerobically (13). Further research conducted in Australia on heliotrine degradation in sheep has resulted in the identification of a single bacterial isolate which is responsible for the metabolism and subsequent detoxification (25).

**Conclusions.** In summary, it is evident that sheep ruminal fluid can metabolize PA under strictly anaerobic methanogenic conditions and that this metabolism is most likely due to small-size ruminal bacteria. These results and others support the hypothesis that PA metabolism is dependent upon an active methanogenic consortium and that nitrate does not appear to serve as an appropriate electron acceptor for PA catabolism under anaerobic conditions. While the removal of protozoa decreases the rate of PA depletion, it is suspected that this may be attributed to the reduction of bacterial numbers which may be adsorbed onto protozoa or plant debris or perhaps due to some synergism between the organisms. The metabolism of PA by sheep ruminal fluid provides another possible example of a natural detoxification mechanism which can render ruminant species resistant

while other similar ruminants are susceptible. It may also serve an integral role upon which the future development of ruminal additives to protect animals or silage inoculants from hazardous plant material occurs.

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